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FOREWORD

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INTRODUCTION

In our proposal we study the role of the class I tyrosine kinase receptor HER-2 in breast cancer. **Our studies are designed to elucidate the contribution of HER-2 to breast cancer growth and progression to hormone independence as well as the development of resistance to treatment with cytotoxic drugs, anti-hormones and HER-2 antibodies.** The HER-2/neu oncogene product is a growth factor receptor of the class I tyrosine kinase receptor family. Members of this family are frequently implicated in tumorigenesis and progression of human cancers [1]. The most common form of activating the transforming potential of HER-2 is gene amplification and receptor over-expression, which is found in breast cancer cell lines as well as in several human adenocarcinomas including breast, ovarian, lung and gastric cancer at a frequency between 20-40% [2-4]. It has been established in numerous clinical studies, that the over-expression of HER-2 in 20-30% of tumor samples of breast cancer patients correlates with a more malignant phenotype, faster progression and a poor prognosis in node-positive patients [5]. There is evidence that the poor patient outcome in HER-2 over-expressing breast cancer patients is linked to a failure of treatment with anti-hormones [6, 7] and cytotoxic drugs [8-10]. However as regards the mechanisms of action of any of these therapies it appears to be unclear and extremely difficult to work out through which target molecule the respective reagents actually act. With this in mind we will use gene specific targeting of HER-2 with ribozyme expression constructs.

OVERVIEW OF THE GOALS

- 1: we study to what extent down-regulation of spontaneous HER-2 expression by targeting with HER-2 ribozymes will affect the *in vitro* and *in vivo* phenotype of HER-2 over-expressing cancer cells.
- 2: investigate whether down-regulation of endogenous HER-2 expression affects the hormone sensitivity of breast cancer cells.
- 3: elucidate the role of HER-2 over-expression in the development of resistance to chemotherapy in cancer cells.
- 4: study whether down-regulation *in vivo* of endogenous HER-2 by targeting with tetracycline regulated HER-2 ribozymes will affect tumor growth in animals.
- 5: investigate if spontaneous expression of a truncated HER-2 receptor coding for the extracellular binding domain (ECD) is involved in the development of resistance to therapy with HER-2 antibodies.

OVERVIEW OF METHODS

For goals 1-4 cell lines are generated with depleted endogenous HER-2 expression levels. This will be achieved through stable transfections with CMV-driven anti-HER-2-ribozyme constructs or through transfections with ribozyme constructs under the control of the regulatable tetracycline promotor system. Growth is assessed by *in vitro* proliferation assays and *in vivo* tumor growth studies in athymic nude mice. Steroid hormones (goal 2) and cytotoxic agents (goal 3) are used in these assays. In goal 5 ribozyme constructs specific for a truncated HER-2 receptor are constructed and transfected into cell lines that over-express the truncated HER-2 receptor.

BODY

Specific Aim 1

Down-regulation of endogenous HER-2 levels in over-expressing cancer cells using ribozyme targeting and the effects thereof on *in vitro* proliferation and modulation of HER-2 associated signal transduction pathways.

Background

HER-2 transfection studies have shown that HER-2 can cause transformation in cultured cells [11]. On the other hand approaches using antisense technology or pharmacological targeting with HER-2 antibodies have provided only limited insight on the role of endogenous HER-2 over-expression in tumor cells. Despite the absence of a high-affinity ligand for HER-2 it is well established that HER-2 can act as an important signaling molecule as a heterodimeric partner with other members of the HER-receptor family.

Work accomplished

1. Generation of cell lines with depleted endogenous HER-2; Effect of HER-2 depletion on tumor cell proliferation and signal transduction.

We stably transfected MDA-MB-361 human breast cancer cells and SK-OV-3 human ovarian cancer cells with CMV driven anti-HER-2-ribozymes. We demonstrated on the RNA level by Northern analysis and on the protein level by FACS analysis that HER-2 expression was reduced by 75 % in 361 cells and to more than 90 % in SK-OV-3 cells (see Figure 1 and Figure 2). In the human breast cancer cell line MCF-7 CMV-driven ribozymes had no effect, but we were able to reduce HER-2 expression by more than 80 % when we expressed the ribozymes under control of the tetracycline regulatable promotor (see Figure 3). Proliferation assays with these cell lines attached to plastic surfaces (96 well assays for up to 7 days; WST-calorimetric assay; Boehringer Mannheim) showed no effects of HER-2 depletion on the doubling time (data not shown). Next we tested the effect of HER-depletion on colony formation in soft agar (standard assay for anchorage-independent growth). Strikingly, we observed that colony formation was dependent on HER-2 in MCF-7 and SK-OV-3 cells (see Figure 4 and Figure 5). MDA-MB-361 could not be tested, since the wildtype cells do not grow colonies in this assay. Finally, we evaluated tumor growth in nude mice. As predicted from the soft-agar results, SK-OV-3 cells with reduced HER-2 levels did not exhibit any significant tumor growth (see Figure 6). This was in striking contrast to 361 cells, where the significant HER-2 reduction had no effect on tumor growth in mice (see Figure 1).

Conclusions

We judge, that the down-regulation of HER-2 by ribozyme-targeting can be extremely efficient. Furthermore, our results demonstrate that HER-2 depletion can exhibit different effects on tumor cell proliferation, depending on the cell line. Finally, the exciting and surprising observation

that colony formation of MCF-7 cells is dependent on HER-2 expression demonstrates that low levels of endogenous HER-2 expression can have a significant impact on breast cancer growth.

Problems and Solutions

we were unable to reduce HER-2 levels in several other cell lines (SK-BR-3; BT-474 and N87) using stable transfections with CMV driven ribozyme constructs. In a next step we will try the tetracycline regulatable promotor to drive ribozyme expression, which is more time-consuming (requires two separate transfection steps), but generally more effective in our hands. We will also try other gene transfer methods such as adenovirus-mediated transduction and ribozyme expression.

2. Effects of HER-2 depletion on Signal transduction

As a model we chose SK-OV-3 cells for the following reasons: the ribozyme-mediated down-regulation of HER-2 was most dramatic (> 90 %) in these cells and they provide a clean model since the Epidermal Growth Factor Receptor (EGFR = HER-1) is the only other HER-receptor subtype expressed in SK-OV-3 cells (see Figure 2). They respond to EGF but not to heregulin (ligand for HER-3 and HER-4). As expected, EGF treatment was followed by tyrosine-phosphorylation of HER-1 and HER-2 in the control cells but not in the HER-2 reduced cells (see Figure 7). Interestingly, the ability of EGF to activate the mitogenic signal transduction cascade (e.g. MAP-Kinase pathway, c-FOS induction) was not dependent on the presence of HER-2 (data not shown). Obviously, activation of HER-1 is sufficient to initiate these responses. On the other hand, despite the activation of the mitogenic cascade, EGF was no longer able to stimulate colony formation in HER-2 reduced cells (see Figure 8). This led us to speculate that the HER-2 reduced cell must die at a higher rate due to apoptosis. Indeed, the percentage of cells undergoing apoptosis spontaneously was significantly increased in HER-2 depleted cells (data not shown; apoptosis was determined by measuring Annexin-V expression at the cell surface by FACS-analysis).

Conclusions

EGF-mediated stimulation of colony formation is dependent on HER-2 expression and phosphorylation. HER-2 activation is required to prevent the cells from undergoing apoptosis.

Next steps

We plan to repeat these studies in other cell lines with different HER-2 receptor expression patterns. We will study the downstream targets of HER-2 necessary to prevent apoptosis (current working hypothesis : differential activation of RAF-1 Kinase leads directly to an altered balance between survival factors such as BCL-2 and death promotors such as Bax or BAD).

Specific Aim 2

To study the role of HER-2 in steroid hormone sensitivity of breast cancer cells.

Background

the proliferation of human breast epithelial cells is regulated by members of the class I receptor tyrosine kinase family as well as steroid hormones. Members of both receptor families are important prognostic factors in breast cancer [12]. Clinical data indicate that over-expression of the HER-2 gene product is associated with an estrogen receptor negative phenotype (ER⁻), a correlation which is also found in most breast cancer cell lines [6]. Additionally, in a recent publication by Slamon et al [7] estrogen dependent MCF7 cells transfected with a HER-2 expression vector became hormone independent followed by a down-regulation of estrogen receptor level and activity. Based on this, we propose that down-regulation of endogenous HER-2 expression by molecular targeting with ribozymes will alter the estrogen sensitivity of HER-2 expressing breast cancer cells.

Work accomplished

Using stable transfections and clonal selection we were able to generate MCF-7 cell lines with down-regulated HER-2 levels (more than 80%; see Figure 3). Furthermore, since the ribozymes are expressed under control of the regulatable tetracycline promotor, addition of either tetracycline or a derivative doxycycline almost completely reversed HER-2 expression to control values. In a first set of experiments we tested the effects of this reversible HER-2 depletion in MCF-7 cells on colony formation in soft agar experiments. We tested spontaneous colony formation, and the effects of 17- β -estradiol in the absence and presence of the pure anti-estrogen ICI 164.384. As mentioned above, we found that spontaneous colony formation of MCF-7 cells was dependent on HER-2 expression (see Figure 4). Interestingly, stimulation of colony formation in the presence of heregulin (ligand for HER-3 and HER-4) was dramatically diminished in the absence of Her-2 expression (see Figure 9). This indicates that most of the heregulin mediated mitogenesis is through heterodimeric complexes containing HER-2 and not through homodimers of HER-4. As a control we stimulated these cells with insulin growth factor I (IGF-I), that stimulates mitogenesis through an unrelated pathway. As expected, the response to IGF-I was not dependent on HER-2 expression, indicating that the ribozyme effect was highly specific for HER-2 (data not shown).

When we tested the hormone sensitivity of these cells, we found, much to our surprise, that depletion of HER-2 rendered MCF-7 cells insensitive to estradiol stimulation (see Figure 10). This fascinating result indicates a direct interaction between HER-2 and estradiol mediated signal-transduction pathways. Furthermore, estradiol mediated growth stimulation seems to be dependent on the presence of the growth factor receptor HER-2. The significance of these findings is further strengthened by the fact that we obtained similar results in the ovarian cancer cell line SK-OV-3, which in contrast to MCF-7 cells express HER-2 at high levels (data not shown).

Conclusions

Depletion of HER-2 prevents estradiol mediated stimulation of colony formation in MCF-7 breast cancer cells. This is in striking contrast to the clinical correlations between HER-2 expression and development of hormone resistance. In light of our data these clinical observations need to be reevaluated and are probably purely correlative and have no mechanistic relevance.

Next Steps

In a next series of experiments we will test if HER-2 depletion in MCF-7 cells regulates the expression levels and/or activity of the estrogen receptor (ER). We will measure ER-levels by ELISA and immunostaining. The activity of the ER will be assessed by co-transfection experiments with an ERE-luciferase reporter plasmid. Finally, we will measure the levels of the progesterone receptor and PS-2 genes, which are regulated by ER. We will then proceed and repeat the experiments in other hormone dependent breast cancer cell lines with depleted HER-2 levels, such as BT-474, T47D and ZR-75-1.

Specific Aim 3

The role of HER-2 over-expression in the development of resistance to treatment with cytotoxic drugs.

Background

There is clinical and experimental evidence suggesting a link between HER-2 over-expression and drug resistance in human breast, gastric, lung and ovarian cancer [8-10], consistent with the association between oncogene over-expression and poor patient outcome. Experimental data so far are either correlative [8] or generated by using HER-2 antibodies in combination with cytotoxic drugs [13]. However, from these experimental approaches it is difficult or impossible to dissect the complex interplay of inhibitory and stimulatory effects on HER-2. Therefore, we propose to use molecular targeting with HER-2 specific ribozymes to down-regulate endogenous HER-2 expression in tumor cells and study the sensitivity to cytotoxic drugs at different HER-2 levels.

Work accomplished

We have generated a panel of useful cell lines with down-regulated HER-2 levels. These are the human breast cancer cell lines MDA-MB-361 and MCF-7, as well as the human ovarian cancer cell line SK-OV-3.

Next steps

We plan to establish dose-response curves with cytotoxic agents representing classes of drugs with distinct mechanisms of action and which are commonly prescribed in chemotherapy of human cancer. We will include doxorubicin/adriamycin (inhibitor of topoisomerase II), 5-fluorouracil (thymidylate synthase inhibitor), cyclophosphamide, cisplatin (DNA-alkylating agents) and taxol (microtubular function inhibitor). We will initially test the drugs on cells attached to plastic surfaces in 96 well plates, and then select the most interesting compounds and selected doses and test these in soft agar experiments.

Specific Aim 4

Down-modulation of HER-2 during different phases of tumor growth.

Background

The strong association between HER-2 over-expression and poor patient outcome has already lead to clinical trials using HER-2 targeted antibody treatment in breast cancer. Yet, it is still unclear if HER-2 is only a marker for poor prognosis or a crucial growth promoting factor at different stages of tumor growth. Therefore we propose to use molecular targeting of HER-2 with ribozymes under the control of a tetracycline regulatable promoter (tet-HER-2-Rz) to study to what extent down-modulation of HER-2 at different time points of tumor development affects the tumor growth in animals of over-expressing cancer cells.

Work accomplished

We were able using a two-step transfection approach to establish two cell lines with down-regulated and tetracycline regulatable HER-2 levels. These cell lines are SK-OV-3 ovarian cancer cells and MCF-7 breast cancer cells. Since MCF-7 cells are an unreliable tumor model, we will initially focus on SK-OV-3 cells. In a first experiment we could demonstrate that depletion of HER-2 led to a cessation of tumor growth. This effect was reversible by tetracycline (see Figure 11). Preliminary data from this experiment suggest that removal of tetracycline treatment (ribozyme was activated) led to a regression of established tumors (see Figure 11). This fascinating result would further strengthen the importance of HER-2 as a target in cancer therapy.

Next steps

We will repeat the experiment with a larger number of animals (20 per group). We will remove the tumors and test for HER-2 expression, proliferation rate (PCNA staining) apoptosis (Tunnel assay) and angiogenesis (CD31 staining). This will allow us to define the molecular mechanisms responsible for the tumor regression. We will try to generate other breast cancer cell lines with depleted and regulatable HER-2 levels.

Problems and Solutions

The generation of cell lines with tetracycline regulatable ribozymes expression and down-modulated HER-2 levels has proven to be difficult in some cell lines. So far we have not been able to generate stable, tetracycline transactivator expressing, derivatives of the SK-BR-3, T47D and MKN7 cell lines. In BT-474 cells the ribozyme effect was not sufficient in a mass-transfected cell population (we are currently selecting clonal cell lines). We will try other gene transfer methods such as electroporation and calcium-phosphate transfection, and in addition we will use different breast cancer cell lines such as ZR-75-1 and MDA-MB-453 cells.

Specific Aim 5

The biological significance of a truncated HER-2/ECD receptor.

Background

Initial investigations by Dr.C.Benz (UCSF) et al [14] described the over-expression of a truncated HER-2 transcript coding for the extracellular ligand binding domain (ECD) in cell lines that are resistant to cytotoxic doses of the HER-2 antibody muAb4D5. This antibody is currently under evaluation in clinical trials and resistance to the antibody treatment in patients has been reported (Dr.C.Benz, personal communication). This prompted us to further evaluate the biological significance of the HER-2/ECD receptor. We propose to study if tetracycline regulated expression of HER-2/ECD in HER-2 over-expressing tumor cells confers resistance to treatment with the 4D5 antibody. Furthermore we will use gene specific targeting of endogenous HER-2/ECD with ribozymes to study if down-regulation of endogenous HER-2/ECD in over-expressing cells restores sensitivity to 4D5 treatment in vitro and in vivo.

Work accomplished

We have generated MCF-7 cells (ECD negative) that over-express the HER-2/ECD receptor in a tetracycline regulatable manner (data not shown).

We have constructed ribozymes specific for the HER-2/ECD transcript and have demonstrated their specificity and activity in vitro cleavage assays (data not shown).

Problems and Solutions

When we transfected the ECD targeted ribozymes into SK-OV-3 and MKN7 cells we found, much to our surprise, that the ribozymes were equally effective against both transcripts, the full length and the truncated ECD transcript (data not shown). Although this prevents us from using this approach, it is in itself a very interesting result , because it proves for the first time that ribozymes are effective in the nucleus and can target hn-RNA. This finding is of great value for ribozyme target selection and considerations of specificity in ribozyme targeting experiments. In order to study the biological significance of the truncated HER-2/ECD receptor we will focus on studying the effects of regulatable ECD over-expression in MCF-7 cells and other cell lines that will be generated.

CONCLUSIONS

We studied the effect of down-regulation of HER-2 expression by ribozyme-targeting on in vitro and in vivo proliferation of cancer cells. We found that colony formation in soft agar was dependent on HER-2 expression in MCF-7 breast cancer cells and SK-OV-3 ovarian cancer cells. The exciting and surprising observation that colony formation of MCF-7 cells is dependent on HER-2 expression demonstrates that already low levels of endogenous HER-2 expression can have a significant impact on breast cancer growth. We studied the molecular mechanisms responsible for these effects and found that heregulin (ligand for HER-3 and HER-4) mediated stimulation of colony formation depends on HER-2 expression in MCF-7 cells and that EGF (ligand for HER-1)-mediated stimulation of colony formation in SK-OV-3 cells is dependent on HER-2. This indicates that HER-2 is the rate limiting receptor in most heterodimeric HER-receptor complexes. Finally, HER-2 activation is not necessary to stimulate mitogenesis but is required to prevent the cells from undergoing apoptosis. Our most striking result is the observation that depletion of HER-2 prevents estradiol mediated stimulation of colony formation in MCF-7 breast cancer cells. This demonstrates the direct cross-talk between the two pathways and furthermore, that activation of a growth factor pathway is required for estradiol mediated stimulation of proliferation.

In conclusion, we have generated a number of initial data and tools for our subsequent studies. We believe that the research has already provided new insights into cancer biology and that some of the data are either submitted for publication or will be in the second year of the award.

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APPENDIX (FIGURES)

Fig. 1

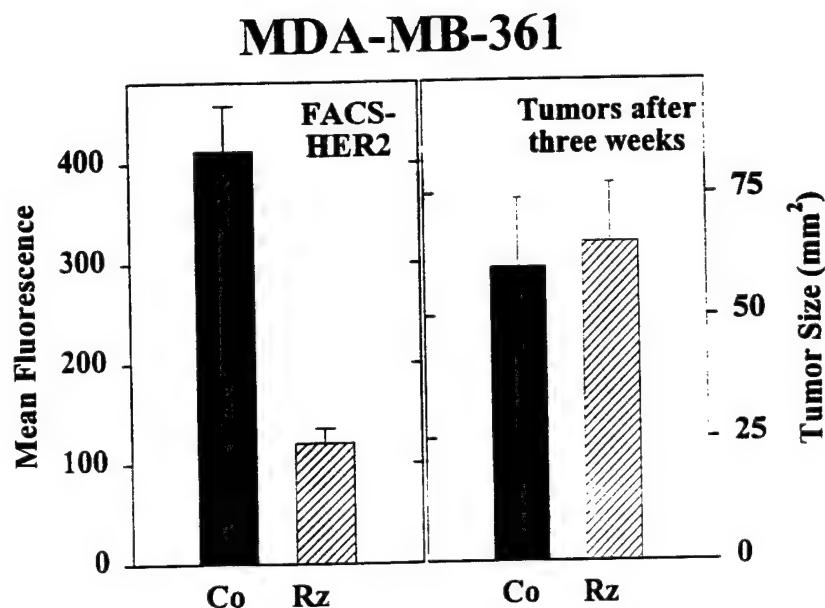


Fig. 1. MDA-MB-361 human breast cancer were stably transfected either with control plasmid (Co) or with a plasmid expressing HER-2 targeted ribozymes under the control of a CMV promotor (Rz). HER-2 levels as quantitated by FACS analysis were reduced by 75 % (left panel). To assess tumor growth in nude mice, control and ribozyme transfected cells were injected at 1×10^6 cells/injection site (6 animals per group; 2 injection sites per animal), and tumor growth was monitored for three weeks (right panel).

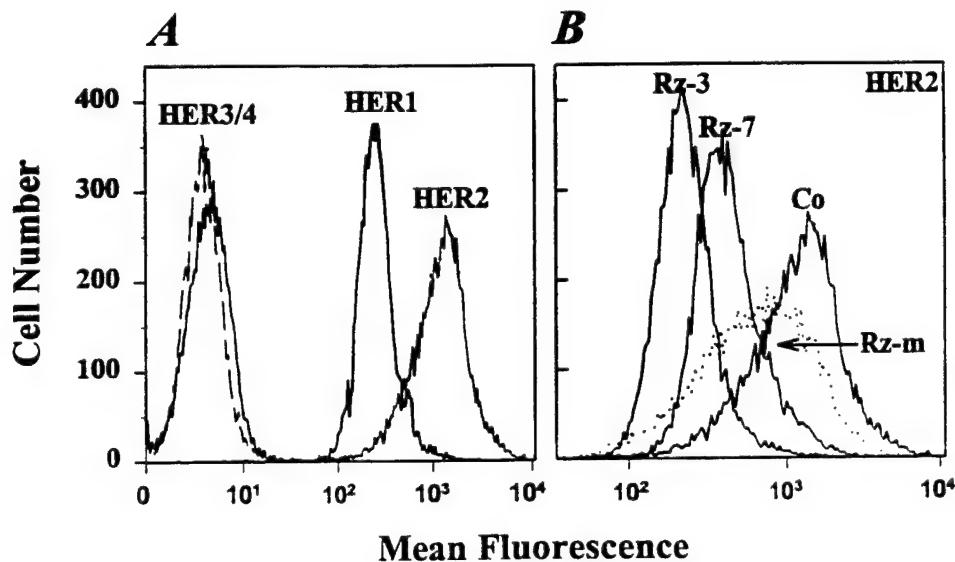
Fig. 2

Fig. 2. Panel A shows the HER-receptor distribution in SK-OV-3 human ovarian cancer cells as assessed by FACS-analysis. SK-OV-3 cells were then transfected with control plasmid (Co), with active HER-2 targeted ribozyme constructs (Rz-3 and Rz-7), or with an inactive mutant control ribozyme (Rz-m). HER-2 levels as measured by FACS-analysis showed a 90 % decrease in HER-2 expression in Rz-3 transfected cells. The inactive control ribozyme had no significant effect.

Fig. 3

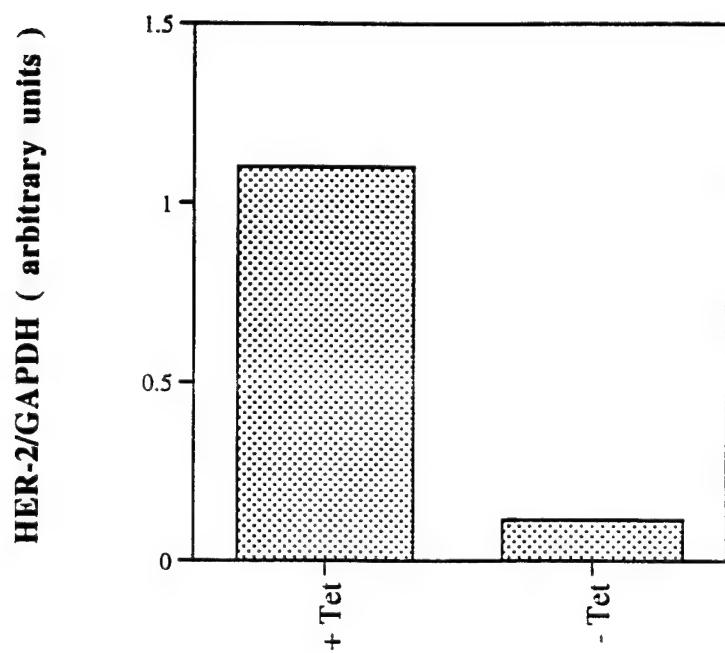


Fig. 3. Densitometry analysis of HER-2 mRNA down-regulation by HER-2 targeted ribozymes in the human breast cancer cell line MCF-7. Ribozymes were expressed under the control of the tetracycline regulatable promotor system, where addition of tetracycline blocks ribozyme expression. The signal intensity of HER-2 mRNA was normalized to the GAPDH mRNA to correct for loading.

Fig. 4

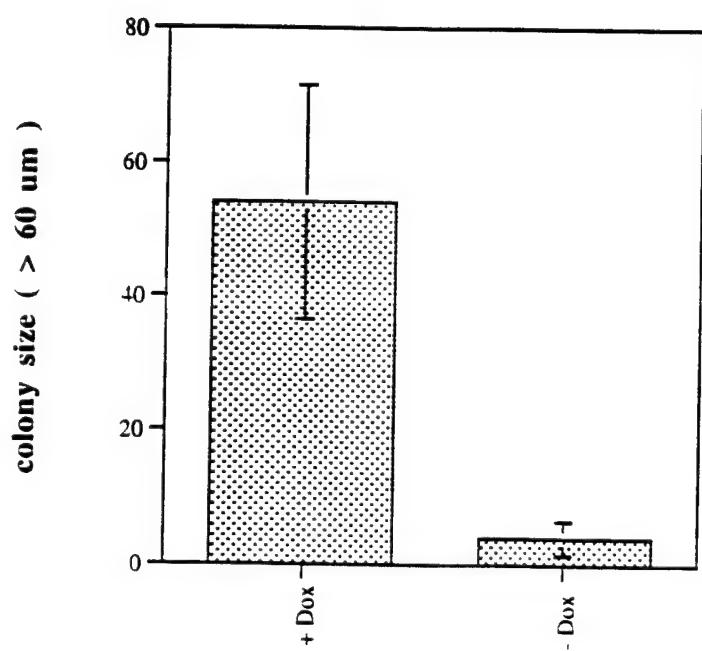


Fig. 4. Down-regulation of HER-2 expression in MCF-7 cells by ribozyme targeting inhibits spontaneous colony formation in soft agar. 10000 cells were plated per 35 mm dish and incubated in the absence or presence of the tetracycline derivative doxycycline at 1 $\mu\text{g}/\text{ml}$. Colonies greater than 60 μm were counted (data shown are mean \pm SD).

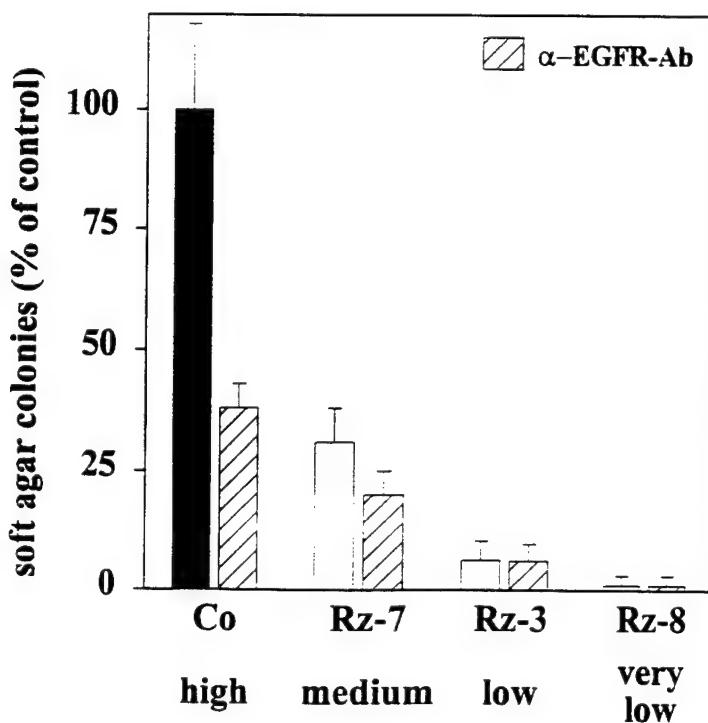
Fig. 5

Fig. 5. Soft agar colony formation in SK-OV-3 cells is dependent on HER-2 expression. SK-OV-3 control cells (Co) or ribozyme transfected cells with HER-2 at medium (Rz-7; ca. 50 % reduction), low (Rz-3; ca. 80 % reduction), or very low levels (Rz-8; more than 90 % reduction) were plated at 10000 cells/35mm dish and incubated for 10 days. Colonies greater 60 μ m were counted (data are shown as % of control; mean \pm SD).

Fig. 6
SK-OV-3

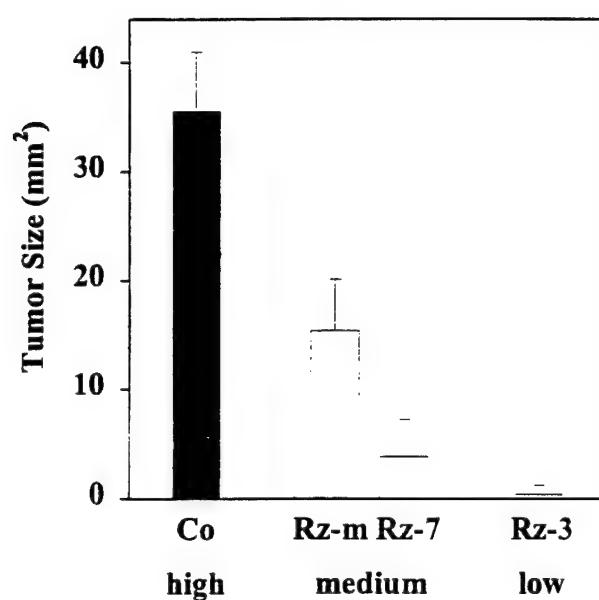


Fig. 6. SK-OV-3 tumor growth in nude mice is dependent on HER-2 expression. To assess tumor growth in nude mice, control (Co) and ribozyme transfected cells with medium (Rz-m; Rz-7) or low HER-2 levels (Rz-3) were injected at 1×10^6 cells/injection site (6 animals per group; 2 injection sites per animal), and tumor growth was monitored for six weeks (data shown are mean \pm SEM).

Fig. 7

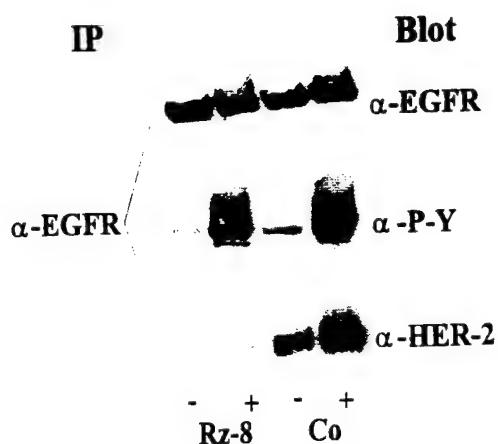


Fig. 7. HER-2 coprecipitates with the epidermal growth factor receptor (EGFR = HER-1) in SK-OV-3 control cells but not in ribozyme transfected cells. Control cells (Co) or ribozyme transfected cells (Rz-8; more than 90 % HER-2 depletion) were treated without (-) or with EGF (+) at 30 ng/ml for 5 minutes. Whole cell lysates were prepared and immunoprecipitated with a polyclonal EGFR antibody. The precipitate was separated on SDS-page and probed with antibodies for EGFR (loading control), anti-phosphotyrosine (α -P-Y) and HER-2 (α -HER-2).

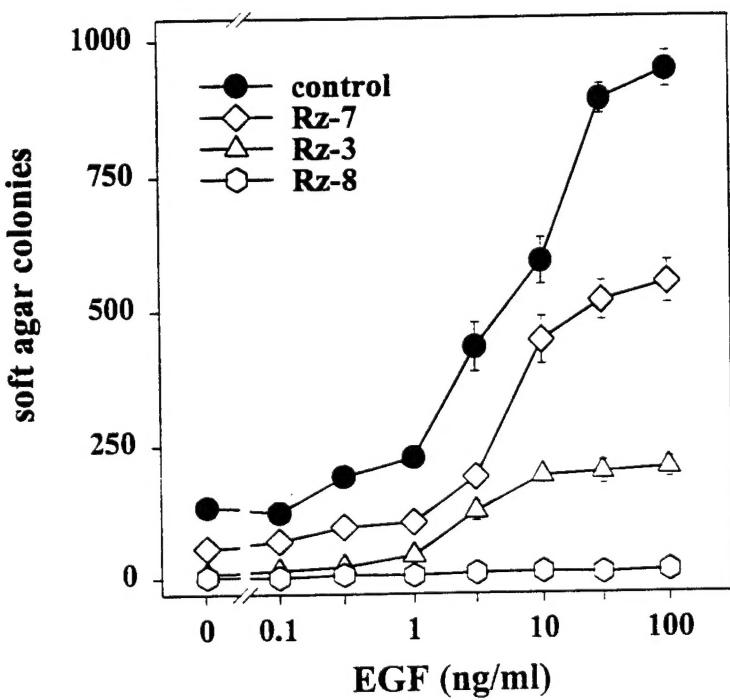
Fig. 8

Fig. 8. EGF-mediated stimulation of colony formation is dependent on HER-2 expression in SK-OV-3 cells. Control cells or cells transfected with ribozymes with HER-2 at different residual levels (Rz-7=medium; Rz-3=low; Rz-8=very low) were plated at 10000 cells / 35 mm dish in the absence or presence of EGF at doses from 0.1 to 100 ng/ml. Colonies greater than 60 μ m were counted after a 10 day incubation period (data shown are mean \pm SD).

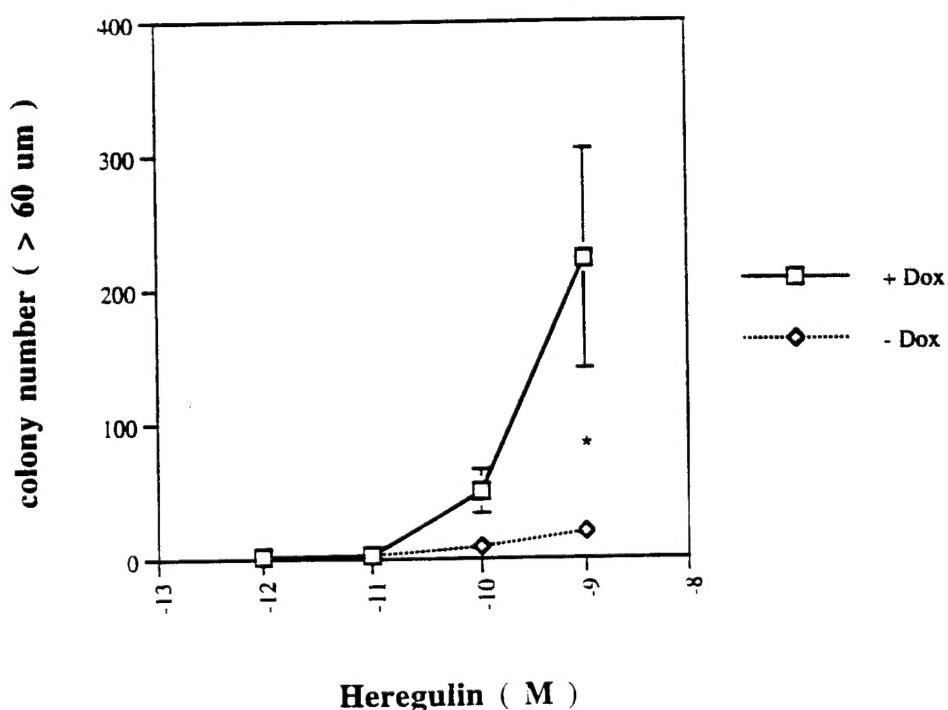
Fig. 9

Fig. 9. Down-regulation of HER-2 expression results in attenuation of heregulin stimulated colony growth in MCF-7 cells transfected with HER-2 targeted ribozymes. MCF-7 cells grown in the absence of doxycycline (- Dox, ribozyme on) or presence of 1 μ g/ml doxycycline (+ Dox, ribozyme off) were treated with heregulin at doses from 10^{-13} to 10^{-8} molar in the presence of the pure anti-estrogen ICI 164 at 10 nM. Colony formation was assessed after a 10 day incubation period (data shown are mean \pm SD; * denotes significant difference; student's t-test, $p < 0.0125$).

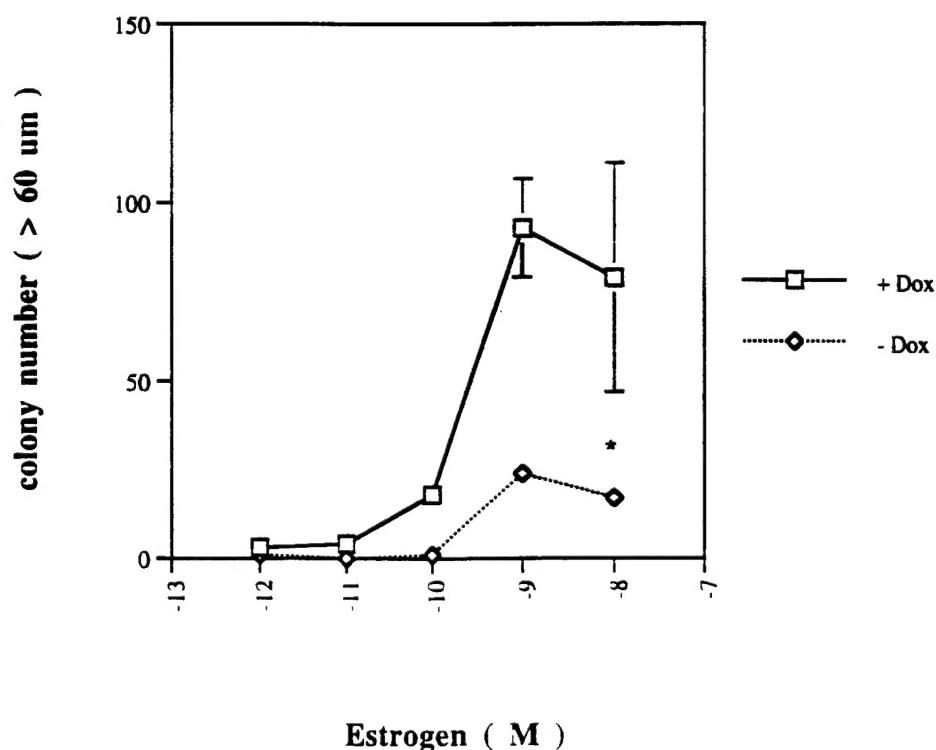
Fig. 10

Fig. 10. Down-regulation of HER-2 expression results in attenuation of estrogen stimulated colony growth in MCF-7 cells transfected with HER-2 targeted ribozymes. MCF-7 cells grown in the absence of doxycycline (- Dox, ribozyme on) or presence of 1 μ g/ml doxycycline (+ Dox, ribozyme off) were treated with estrogen at doses from 10^{-13} to 10^{-7} molar in the presence of the pure anti-estrogen ICI 164 at 10 nM. Colony formation was assessed after a 10 day incubation period (data shown are mean \pm SD; * denotes significant difference; student's t-test, $p < 0.0007$).

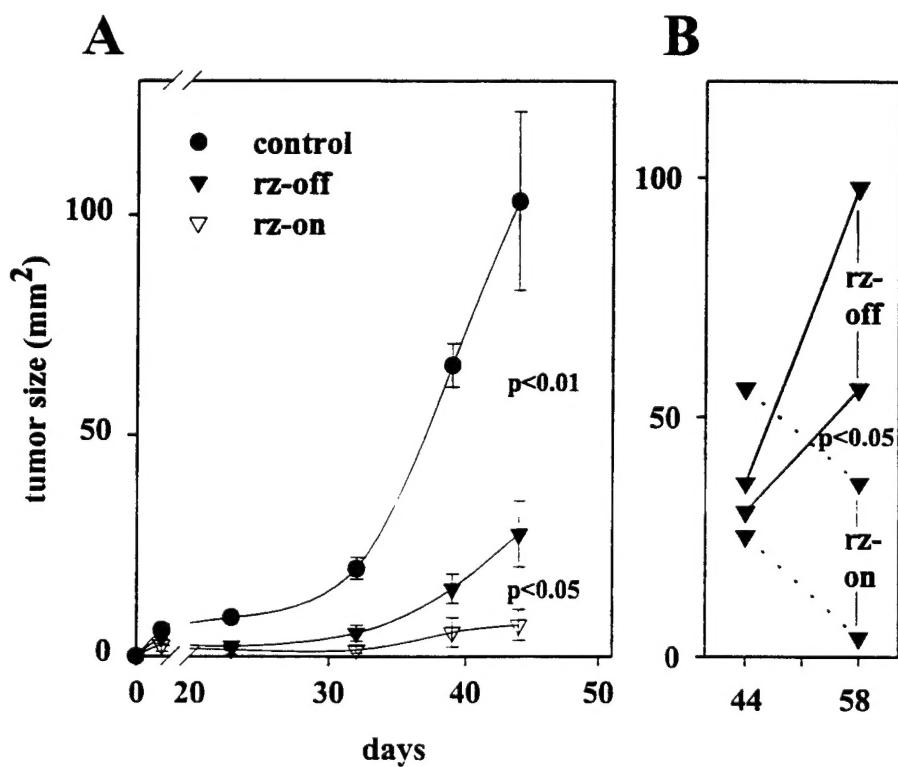
Fig. 11

Fig. 11. SK-OV-3 tumor growth curves in nude mice. One million cells were inoculated (3 mice per group; 2 injection sites per group), and tumor growth was monitored for six weeks in control cells (filled circles) or ribozyme transfected cells in the presence (rz-off; filled triangles) or absence (rz-on; open triangles) of tetracycline treatment (A). After 44 days growth curves of the individual tumors in the absence (rz-on; dotted lines) or continued presence of tetracycline (rz-off; solid lines) were monitored for another two weeks (B).